Bioorganic & Medicinal Chemistry Letters 19 (2009) 5764–5768

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Simple dihydrosphyngosine analogues with potent activity against MDR-*Mycobacterium tuberculosis*

Esther del Olmo [a,*], Gloria María Molina-Salinas [b], Ricardo Escarcena [a], Mario Alves [a], José L. López-Pérez [a], Rogelio Hernandez-Pando [c], Salvador Said-Fernández [b,*], Arturo San Feliciano [a]

[a] *Departamento de Química Farmacéutica, Facultad de Farmacia, CIETUS, Universidad de Salamanca, Spain*
[b] *Laboratorio de Micobacteriología. Centro de Investigación Biomédica del Noreste, IMSS, Monterrey, Mexico*
[c] *Departamento de Patología. Instituto Nac. de Ciencias Médicas y Nutrición 'Salvador Zubirán', Tlalpan, Mexico*

ARTICLE INFO

Article history:
Received 17 June 2009
Revised 29 July 2009
Accepted 30 July 2009
Available online 3 August 2009

Keywords:
Dihydrosphingosine
Ethambutol
Aminoalcohols
Diamines
Mycobactericidal
Mycobacterium tuberculosis
H37Rv strain
MDR strain

ABSTRACT

Fifteen dihydrosphingosine analogues have been synthesized and tested in vitro against *Mycobacterium tuberculosis* (*MTB*). Two ether (**3** and **4b**) and one diamine (**8b**) derivatives have displayed high mycobactericidal potency, with similar MIC values of 1.25 μg/mL, against the virulent strain H37Rv, as well as against a clinical isolate resistant to the five first-line anti-TB drugs. The three compounds, tested on other eleven cultured *MTB* strains with different multi-drug-resistance (MDR) patterns, retained their MIC values for most strains, or even lowered it, as in the case of compound **4b,** which, assayed on strain No. 332, also resistant to all first-line anti-TB drugs, attained the MIC value of 0.78 μg/mL.

Tuberculosis (TB), mainly caused by *Mycobacterium tuberculosis* (*MTB*), has been widespread since ancient times and remains a worldwide major health problem. TB morbidity, with 8–9 million new infections per year, and its mortality, with 3 million deaths annually,[1] represents the largest numbers of incidence and of human deaths attributable to a single etiologic agent. Mycobacteria are able to survive in a latent state in infected individuals, thereby serving as a reservoir, waiting for the opportune reactivation.[2] According to World Health Organisation (WHO) estimation, 2 billion people, almost one-third of the world's population, are believed to be infected with *MTB*.[3]

After several decades of decline, TB is currently rising, mainly due to co-infection with HIV (AIDS) in immunocompromised patients. It is estimated that 15 million people are simultaneously infected with HIV and *MTB*, and around 0.5 million of the 1.36 million HIV-associated deaths in 2007 were directly attributable to TB.[4] An additional contributing factor is the continued emergence of new MDR–*MTB* strains,[5] often associated with poor compliance to treatments and, since 2006, to the officially recognized appearance of *MTB* strains with extended resistance (XDR-*MTB*),[6] now spread to more than 50 countries.[7] Furthermore, TB treatment protocols are prolonged (six months), complex and rather expensive and in the majority of cases end in the poor patient compliance.

Due to the facts mentioned above and to TB latency new anti-TB drugs and better therapeutic strategies against TB are urgently needed. New drug candidates should shorten the standard treatments and be sufficiently effective against MDR-TB. Though several compounds are currently in advanced phases of clinical assay, during some forty years no new compounds have been introduced in the market for TB treatment. However, in recent years an emphasized research activity in the development of new TB drugs has been produced. Some compounds are, at present, in clinical development, while others are being investigated pre-clinically in an attempt to discover new molecules for a target-based treatment of TB.[8] Crossing the vast, heavy and most complex polypeptide–polyglycoside–polylipidic mycobacterial wall is one of the major problems these compounds have to overcome.[9]

Sphingosine (SPH, Fig 1) is a basic component of sphingolipids and ceramides. It is biosynthesized from serine and palmitic acid through its saturated precursor dihydrosphingosine (DSPH). Sphingosine 1-phosphate (S1P), the activated form of SPH, is involved in a wide spectrum of biological processes, including Ca^{2+} mobilization, cell growth, differentiation, motility and cytoskeleton organization.[10] Most interestingly it has been reported that the roles of

* Corresponding author. Fax: +34 923294515.
E-mail address: olmo@usal.es (E. del Olmo).

0960-894X/$ - see front matter © 2009 Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2009.07.147

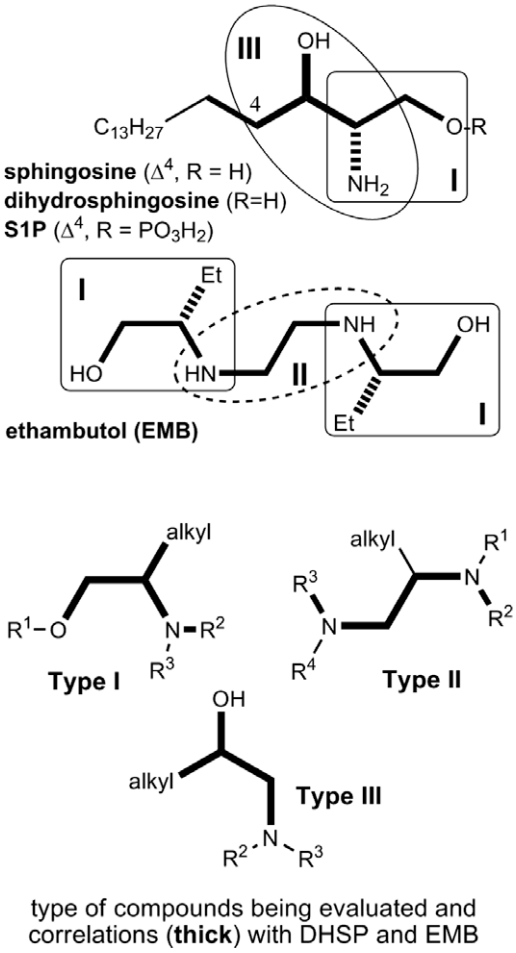


Figure 1. The structural basis of this research.

SPH, S1P, ceramides and other lipid components on *MTB* containing endosomes lead to lysosome activation in the *MTB*-infected cell.[11] This observation could support the use of sphingoanalogues in the configuration of effective drug regimes for attacking the human *MTB* reservoir, the inveterate latent TB, affecting one third of the planet's population.

From the structural point of view, SPH and DSPH contain, as a pseudo-symmetric functional arrangement, a duplicate aminoethanol (hydroxyethylamine) fragment also present in duplicate though separated by an ethylene bridge, in the structure of one of the first-line anti-TB drugs, ethambutol (EMB, Fig. 1). In addition, EMB contains the central ethylenediamine bridge, which is also necessary for its antimycobacterial activity. Studies of the mechanism of action of EMB have revealed its ability to interfere with the integrity of the mycobacterial cell wall through the inhibition of arabinogalactan biosynthesis,[12] though other possible pathways have also been proposed.[13]

These facts prompted us to consider the evaluation, against *MTB,* of a selected number of linear aminoethanol and ethylenediamine derivatives with the objective of discovering new potential anti-TB agents. As can be seen in Fig. 1 the compounds selected for evaluation (**types I**, **II** and **III**) contain fragments closely related to DSPH and to EMB. In this preliminary in vitro evaluation we have only included saturated and structurally simplified racemic compounds, chemo-modulated mainly through common and simple alkylation/acylation of functional groups, in order to configure false DSPH analogues containing either original or masked pharmacophoric fragments of EMB to provide more polar and more soluble or latentized forms.

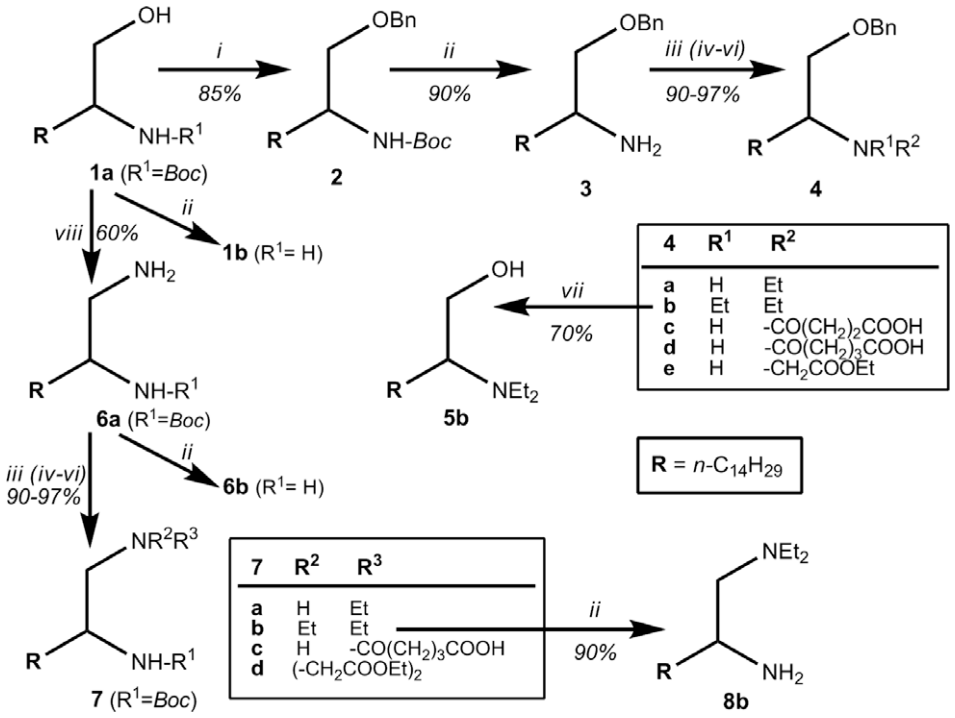
For several years our research group has been working on the synthesis of this type of family of compounds and has reported on the results derived from several bioactivity tests, including phospholipase A2 inhibition, anti-inflammatory and anti-parasitic evaluations.[14a–e] Further support for the hypothetical antimycobacterial activity of these compounds were found in recent descriptions of the potential interest of NSAID (through inhibition of prostaglandin biosynthesis)[15] and immune-modulation (through induction/inhibition of selective cytokines)[16] drugs for anti-TB therapy. Both aspects, respectively represented by PLA_2 inhibition,[14a] lymphoproliferation inhibition[17] and vaccination adjuvation[18] have been documented previously by us for some substances belonging to the series included in this research. Experimental results from such diverse bioactivity evaluations reported in our previous papers, also support the selection of the palmitic size (tetradecyl side-chain) as the most promising for a better antimycobacterial activity of these compounds, while facilitating comparisons on the influences due to the range of functionalities implemented in the structures.

The synthesis of several series of lipidic aminoalcohols and diamines has been reported previously by us.[14] Here we include only a brief description of the procedures followed to obtain those compounds tested in this research (Scheme 1). In summary, the Boc-protected aminoalcohol **1a**, used as starting material, was transformed into its benzyl ether **2**. Boc-deprotection in acid conditions gave the benzyl ether-amine **3**. This intermediate **3**, was monoalkylated and transformed into the secondary amines **4a** and **4e,** by treatment with either ethyl bromide or ethyl bromacetate, respectively. Amides **4c** and **4d** were obtained by acylation with succinyl and glutaryl anhydrides, respectively. The tertiary amine **4b** resulted from dialkylation of **3** with ethyl bromide excess, while catalytic hydrogenolysis of the benzyl ether **4b** led to the free primary alcohol **5b**.

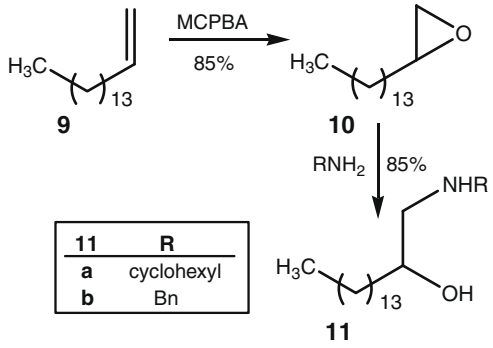
Diamine derivatives were also prepared from compound **1a**. The hydroxyl group was first mesylated, then transformed into the corresponding azide and further reduced to the diamine **6a**. Treatment of **6a** with either one or two equivalents of ethyl bromide gave predominantly compounds **7a** and **7b**, respectively. The reaction of **6a** with glutaric anhydride yielded compound **7c**, while its alkylation with an excess of ethyl bromacetate led to the disubstituted amine **7d**. Compound **7b** was Boc-deprotected with acid to yield the diamine **8b**.

1-Aminohexadecan-2-ol derivatives were prepared from 1-hexadecene (**9**), through epoxidation with *meta*-chloroperbenzoic acid, followed by oxirane opening with cyclohexylamine or benzylamine leading to aminoalcohols **11a** and **11b**, respectively, in good yields (Scheme 2). The inclusion of only two compounds with inverted functionality in this study, as well as the selection of benzylamine and cyclohexylamine, was due to several reasons. According to the structure correlations shown in Figure 1 and to the fact that the primary alcohol of SPH constitutes the actual activating phosphorylation target, the expectancy of activity for compounds containing the inner fragment (**III**) of DSPH, with the secondary alcohol, should be lower than that expected for those containing the outer (**I**) fragment. The benzyl group as an N-substituent had not been considered in compounds of types **4**–**8**. It could easily be removed and replaced by any other group, thus opening the route to the needed or desired chemodiversity. Moreover, the interest of the cyclohexyl fragment can be focused on its own secondary nature which could mimic the isopropyl group, also absent in compounds **4**–**8** and, due to its potential free rotation around the N-cyclohexyl bond, it could also mimic *t*-Bu, adamantanyl and bulkier radicals.

Fifteen compounds of those represented in Schemes 1 and 2, being four aminoalcohols, five aminoethers and six diamine derivatives, were selected and submitted to the in vitro MABA anti-*MTB* assay.[19–21] The MIC values found are shown in Table 1, along with the



Scheme 1. Synthesis of some dihydrosphingoanalogues. Reagents and conditions: (i) BnCl/NaH/DMF; (ii) HCl/THF; (iii) EtBr/Et_3N/DMF; (iv) glutaric anhydride/CH_2Cl_2; (v) succinic anhydride/CH_2Cl_2; (vi) ethyl bromacetate/CH_2Cl_2; (vii) H_2/Pd–C/AcOH; (viii) (a) MsCl/Et_3N/CH_2Cl_2; (b) NaN_3/DMF; Pd–C/THF/$NaBH_4$/MeOH.



Scheme 2. Synthesis of 1-aminohexadecan-2-ol derivatives from 1-hexadecene.

value for EMB, for a close structure–activity comparison and the value for rifampicin (RMP) as reference of another first-line pharmaceutical, a very effective and potent anti-TB drug, which acts through RNA-polymerase inhibition. Due to large differences in molecular weight between compounds and standard drugs, MIC values are expressed in μM units rather than in μg/mL, most commonly found in the literature reporting screening anti-*MTB* results, to provide adequate comparisons of antimycobacterial potencies. Boldface numbers in each column represent MIC values lower than those corresponding to EMB, the reference drug, structurally close to the compounds evaluated. Antimicrobial potencies relative to EMB (P_E), calculated for both, sensitive and MDR strains by means of the equation $P_E = MIC_E\,(\mu M)\,/MIC_{COMP}(\mu M)$ are also included.

As can be observed the compounds of the three different series were, in general, active against sensitive and MDR–*MTB* and better against the MDR strain in comparison with the reference drug EMB. It can also be seen that both **Type I** and **Type II** sub-series contain representative compounds which were more potent than EMB. Nevertheless, such considerations cannot be extended to **Type III** derivatives due to the reduced number of compounds being evaluated and to the differences in substituents with respect to the other sub-series. Furthermore, compounds of **Type I** and **Type II** seem to behave in an almost parallel manner, though within all the results obtained only one direct comparison can be made. In this case, the double primary 1,2-diamine **6b** (12.6 μM) resulted one-dilution-level more potent than the free 2-aminopalmitol **1b** (24 μM).

One of the most interesting facts observed is related to the analogy of the relative anti-*MTB* potencies calculated for compounds **3**

Table 1
Antimycobacterial effect of dihydrosphingoanalogues on sensitive (H37Rv) and MDR (CIB99) MTB strains[a]

Type	Compd	H37Rv		CIB99	
		MIC(μM)	P_E[b]	MIC(μM)	P_E[b]
I 2-amino-1-alkanol	**1a**	84	0.12	84	>5
	1b	24	0.41	49	**>16**
	3	**3.6**	**2.8**	**3.6**	**>43**
	4a	16.6	0.6	16.6	>9.5
	4b	**3.1**	**3.2**	**3.1**	**>50**
	4c	140	0.07	140	>1.1
	4d	68	0.14	68	>2.3
	4e	72	0.13	72	>2.2
	5b	20	0.5	40	>3.9
II 1,2-alkane-diamine	**6a**	30	0.3	30	>5.2
	6b	12.6	0.8	12.6	**>12**
	7a	32	0.3	32	>4.9
	7b	15.2	0.65	15.2	**>10**
	7c	133	0.07	133	>1.2
	7d	59	0.17	59	>2.7
	8b	**4.0**	**2.5**	**8.0**	**>19**
III 1-amino-2-alkanol	**11a**	33	0.30	11	**>14**
	11b	36	0.28	72	>2.2
Ref. drugs	**EMB**	9.9	**1**	>158	**1**
	RMP	0.152	**650**	>2.43	~**65**

[a] CIB99 is a complete SIREP-resistant *MTB* strain.[19]
[b] Anti-*MTB* **p**otency, relative to that of **e**MB. [$P_E = MIC_E\,(\mu M)/MIC_{COMP}\,(\mu M)$].

(P_E value >43) and **4b** (>50) and that estimated for the highly potent anti-TB drug RMP (P_E ~65) in the case of the MDR strain. Other P_E bold-face values in the CIB99 column correspond to compounds with antimycobacterial potency one-order or higher than that of EMB against the MDR strain. Another observation relates to the negative influence of the Boc-protecting group on the activity, which can be observed either against the sensitive as the MDR strain, for the pairs of compounds **1a/1b** (84 vs 24 μM, respectively, for H37Rv MICs), **6a/6b** (30 vs 12.6 μM) and **7b**/**8b** (15.2 vs 4.0 μM). On the other hand, the presence of the benzyl ether moiety compares positively with its absence in the alcohol derivatives tested, as can be seen for the pairs of compounds **3/1b** (3.6 vs 24 μM) and **4b/5b** (3.1 vs 20 μM).

Relating to substituents attached to the amine groups, the negative influence mentioned above for *N*-Boc protection, can be extended to hemisuccinyl and hemiglutaryl amides belonging to both the aminoalcohol and the diamine series, with respect to unsubstituted, mono- or dialkylated amines. This can be seen for **Type I** amides **4c**, **4d** (140, 68 μM, respectively) in comparison with compounds **3**, **4a**, **4b** (3.6, 16.6, 3.1 μM) and **Type II** amide **7c** (133 μM) as compared with compounds **6b**, **7a**, **7b** (12.6, 32, 15.2 μM). The analysis of the above values, in brackets, also demonstrates that unsubstituted and dialkylated amines are rather better than those monosubstituted analogues. Finally, comparison of anti-*MTB* potencies of ethyl (**4a**, **7b**; 16/15.2 μM) or methoxycarbonylmethyl (**4e**, **7d**; 72/59 μM) alkylated amines give preference for the first group. Nevertheless, more compounds with other substituents of different size and constitution should be prepared and tested to attain a better definition of their influence on anti-*MTB* activity.

Confirmatory assays were carried out with the most potent antimycobacterial compounds **3**, **4b** and **8b**[22] on eleven *MTB* strains with different MDR profiles. The results are reported in Table 2 and confirmed the potentiality of two of these compounds against multi-resistant *MTB* strains. As can be seen, while the diamine derivative **8b** showed a one-order reduced potency, compounds **3** and **4b** presented a sustained activity against most MDR strains. Thus, compound **3** was similarly effective on strains No.401 and No.366, resistant to four of the first-line drugs, and on strain No. 411, resistant to all of them. Similarly, the diethylamine derivative **4b**, was effective on strain No. 363, I,E-resistant, on strain No. 366, resistant to four TB drugs and on strain No. 535, resistant to all of them. Interestingly, compound **4b**, with an MIC value of 1.9 μM, resulted even more effective against strain No.332, resistant to all the first-line anti-TB drugs. It is interesting to note that compound **8b** was much more active against CIB99 than to the other MDR strains included in Table 2. It is well known that resistance to anti-mycobacterial agents is due to the presence of mutations in one or more genes.[23] Furthermore, within one gene several mutations could appear which would produce different grades of resistance against the same compound.[24] Thus, possibly one or more mutations could be present in CIB99, but not in the other tested strains, making CIB99 more susceptible to **8b**; or, reciprocally, the other strains could have a particular mutation absent in CIB99 inducing resistance to the diamine **8b**, but not affecting the susceptibility to aminoalcohol derivatives **3** and **4b**. Nevertheless, this matter would need the characterization of the complete genome of all the strains used in the present study.

In summary, representative compounds of the two main types (**I** and **II**) of the dihydrosphingoanalogues tested in this study have shown a fair in vitro anti-*MTB* activity against sensitive and MDR strains and their potential utility as anti-TB drugs seems to merit further consideration. On the basis of their behavior against MDR strains compounds **3** and **4b** have been sent for their acute toxicity to be evaluated (LD_{50}), before carrying out in vivo assays, necessary to ascertain their real anti-TB effectiveness in infected mice. Molecular modeling calculations, ADME and toxicity prediction, involving compounds with some changes in functionalities and side-chain sizes of analogues, as well as preparative synthetic work, aiming to extend the evaluation to a larger number of compounds, are being performed; particularly for compounds of **type III**. Other studies to define the precise target(s) for these compounds in the mycobacteria, oriented to establish the actual mechanism of action, are also being planned.

Table 2
Antimycobacterial profile of compounds **3**, **4b** and **8b** on MDR–*MTB* strains

MTB strain No.[a]	Resistance profile S I R E P	MIC (μM)		
		3	**4b**	**8b**
528	R R R R R[b]	18	15.5	40
160	R R R R R	9.0	7.7	40
401	R R R S R	**4.5**	7.7	40
494	R R R S R	9.0	7.7	40
411	R R R R R	**4.5**	7.7	40
331	R R R R R	9.0	15.5	40
429	S R R R R	9.0	7.7	40
366	R R R R S	**4.5**	**3.9**	20
363	S R S R S	18	**3.9**	40
332	R R R R R	36	**1.9**	40
535	R R R R R	18	**3.9**	40
H37Rv	S S S S S	**3.6**	**3.1**	**4.0**

SIREP: **S**treptomycin, **I**soniazid, **R**ifampicin, **E**thambutol, **P**yrazinamide.
[a] Reference characterisation No. given at the CIBIN-IMSS Research Centre.
[b] **R**: resistant and **S**: sensible, to the corresponding drug above.

Acknowledgements

Collaborative work performed under the auspices of *Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo* (CYTED), Project X.11: PIBATUB. Financial support came from Spanish Minister of Science (Grant: AGL-2005-02168 GAN). SSF and GMMS wish to thank the Instituto Mexicano del Seguro Social for financial support, which allowed the development of antituberculosis bioassays. We would like to thank Mr. G. H. Jenkins for his help with the English version of the ms.

References and notes

1. http://www.who.int/tb/publications/global_report/2009/en/index.html
2. Andersen, P.; Doherty, T. M.; Pai, M.; Weldingh, K. *Trends Mol. Med.* **2007**, *13*, 175.
3. http://www.who.int/features/factfiles/tb_facts/en/index1.html
4. WHO report 2009. *Global Tuberculosis Control: Epidemiology, Strategy, Financing,* Geneva, p 10.
5. http://www.who.int/tb/challenges/mdr/faqs/en/index.html
6. Gandhi, N. R.; Moll, A.; Sturm, A. W.; Pawinski, R.; Govender, T.; Lalloo, U.; Zeller, K.; Andrews, J.; Friedland, G. *Lancet* **2006**, *368*, 1575.
7. http://www.who.int/tb/challenges/xdr/en/index.html
8. Ahirrao, P. *Mini-Rev. Med. Chem.* **2008**, *8*, 144.
9. Minnikin, D. E.; Kremer, L.; Dover, L. G.; Besra, G. S. *Chem. Biol.* **2002**, *9*, 545.
10. Lynch, K. R.; Macdonald, T. L. *Biochem. Biophys. Acta* **2008**, *1781*, 508.
11. Russell, D. G. *Nat. Cell Biol.* **2003**, *5*, 776; Anes, E.; Kühnel, M. P.; Bos, E.; Moniz-Pereira, J.; Haberman, A.; Griffiths, G. *Nat. Cell Biol.* **2003**, *5*, 793.
12. Takayama, K.; Kilburn, K. O. *Antimicrob. Agents Chemother.* **1989**, *33*, 1493; Bhowruth, V.; Alderwick, L. J.; Brown, A. K.; Bhatt, A.; Besra, G. S. *Biochem. Soc. Trans.* **2008**, *36*, 555.
13. (a) Cole, A.; May, P. M.; Williams, D. R. *Agents Actions.* **1981**, *11*, 296; (b) Solecki, T. J.; Aviv, A.; Bogden, J. D. *Toxicology* **1984**, *31*, 207; (c) King, A. B.; Schwartz, R. *J. Nutri.* **1987**, *117*, 704.
14. (a) Lucas, R.; Ubeda, A.; Paya, M.; Alves, M.; Olmo, E.; Lopez, J. L.; San Feliciano, A. *Bioorg. Med. Chem Lett.* **2000**, *10*, 285; (b) Olmo, E.; Alves, M.; Lopez, J. L.; Inchaustti, A.; Yaluff, G.; Rojas de Arias, A.; San Feliciano, A. *Bioorg. Med. Chem Lett.* **2002**, *12*, 659; (c) Olmo, E.; Macho, M.; Alves, M.; Lopez, J. L.; Banaua, F.; Muñoz, E.; San Feliciano, A. *Bioorg. Med. Chem Lett.* **2002**, *12*, 2621; (d) Lucas, R.; Alves, M.; Olmo, E.; San Feliciano, A.; Paya, M. *Biochem. Pharmacol.* **2003**, *65*, 1539; (e) Lucas, R.; Villamon, E.; Paya, M.; Alves, M.; Olmo, E.; Gozalbo, D.; Gil, M. L. *FEMS Immunol. Med. Chem.* **2004**, *40*, 239.
15. Rangel, J.; Estrada, I.; Garcia, M. I.; Aguilar, D.; Marquez, R.; Hernández-Pando, R. *Immunology* **2002**, *106*, 257.

16. Rook, G. A. W.; Lowrie, D. B.; Hernández-Pando, R. *J. Infect. Dis.* **2007**, *196*, 191.
17. Olmo, E.; Plaza, A.; Muro, A.; Martínez-Fernández, A. R.; Nogal-Ruiz, J. J.; López-Pérez, J. L.; San Feliciano, A. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 6091.
18. (a) Uribe, N.; Siles-Lucas, M.; López-Abán, J.; Esteban, A.; Suarez, L.; Martínez-Fernandez, A. R.; Olmo, E. del.; Muro, A. *Vaccine* **2007**, *25*, 4533; (b) Siles-Lucas, M.; Uribe, N.; López-Abán, J.; Vicente, B.; Orfao, A.; Nogal-Ruiz, J. J.; San Feliciano, A.; Muro, A. *Vaccine* **2007**, *25*, 7217; (c) López-Abán, J.; Nogal-Ruiz, J. J.; Vicente, B.; Diez-Baños, P.; Hillyer, G. V.; Martínez-Fernández, A. R.; San Feliciano, A.; Muro, A. *Vet. Parasitol.* **2008**, *153*, 176.
19. *Mycobaterial characterisation*: The H37Rv *MTB* strain (ATCC cat. No. 27294) resulted sensitive to all first-line anti-TB drugs, S, I, R, E and P. The CIBIN/UMF28:099 (CIB99) strain was isolated from a patient with advanced lung TB and characterised in the Mycobacteria laboratory of *Centro de Investigaciones Biomédicas del Noreste-IMSS*, Monterrey, N.L., Mexico. This strain was resistant to all the above mentioned drugs. Its MIC values for S, I, R and E, determined by MABA, were respectively higher than 13.8, 15.0, 1.2 and 157 μM. Using the conventional BACTEC 460-radiometric system (Bactec 460).[25] This strain was resistant to the critical antimicrobial concentration of S, I, R, E and P. Other MDR–*MTB* strains (Table 2) were characterised similarly.
20. *Assay:* The modified Microplate Alamar Blue Assay (MABA)[21] was used to determine the anti-*MTB* activity. Briefly, solutions of compounds (2 mg/mL, DMSO), standard drugs, RMP (1 μg/mL, DMSO), EMB·HCl (2 mg/mL, H_2O), were sterilised by filtration (PTFE acrodiscs, 0.22 μm pore size, Millipore Co., Bedford, MA, USA). Std drugs were divided in 0.5 mL aliquots, and stored at −70 °C until used, while the compounds were assayed immediately. Deionised sterile water (200 μL) was dispensed in the perimeter wells to diminish evaporation. The inner wells were divided in lanes of 6 to perform the assays. 100 μL of a 1:50 bacterial suspension (having about 6×10^6 CFU/mL, Mc Farland std 1) in Middlebrock 7H9-OADC enriched broth (Becton Dickinson and Co., Sparks, MD, USA) were added to each testing well. In the 6-well lanes, compound and standard drug solutions were two-step serially diluted with 7H9-OADC medium (100 μL per well). Evaluations were performed on dilutions within the ranges: 1–100 μg/mL (compounds), 0.062–2.0 μg/mL (RMP) and 1.0–32 μg/mL (EMB). A blank (100 μL of medium), and a positive mycobacterial growth control (100 μL of 1:50 bacterial suspension) were included. After incubation (37 °C for 5–7 days in 5% CO_2 atmosphere, into plastic O_2 permeable sealed bag), 32 μL of freshly prepared 20:12 mixture Alamar Blue plus 10% Tween-80 (v/v) were added to each well. The plates were re-incubated at 37 °C for 24 h. Pink and blue colours indicated mycobacterial growth or its absence, respectively. The MIC value for a tested compound was assigned to that of the first blue well. Destruction of mycobacteria in the first blue well was confirmed microscopically.
21. Molina-Salinas, G. M.; Ramos-Guerra, M. C.; Vargas-Villarreal, J.; Mata-Cárdenas, B. D.; Becerril-Montes, P.; Said-Fernández, S. *Arch. Med. Res.* **2006**, *37*, 45.
22. *Analytical data for compound* **3**: white crystal, mp: 72 °C. IR ν_{max}: 3379, 293, 1589, 1459, 1362, 1204, 900 and 736 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.33 (5H, m, Ar); 4.53 (2H, s, CH_2–Ar); 3.46 (1H, dd, $J_1 = 8.9$, $J_2 = 3.6$ Hz, H-1_A); 3.23 (1H, dd, $J_1 = 8.9$, $J_2 = 7.9$ Hz, H-1_B); 2.99 (1H, m, H-2); 1.25 (26H, m, $(CH_2)_{13}$); 0.91, (3H, t, $J_1 = 6.1$, CH_3).). ^{13}C NMR (50.3 MHz, $CDCl_3$): δ (ppm) 138.8, 128.4, 127.7, 75.8, 73.2, 51.1, 34.2, 31.7, 29.7, 29.4, 26.1, 22.7, 14.2. HRMS: Calcd: 348.3244 (M^++H); found: 348.3261. Anal. Calcd for $C_{23}H_{41}NO$: C, 79.48; H, 11.89; N, 4.03. Found: C, 79.46; H, 11.83; N, 4.05.
Analytical data for compound **4b**: Yellow oil. IR ν_{max}: 2923, 2854, 1696, 1459, 1371, 1205, 1107, 906 and 734 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ (ppm) 7.33 (5H, m, Ar); 4.51 (2H, s, CH_2–Ar); 3.56 (1H, dd, $J_1 = 9.6$, $J_2 = 5.7$ Hz, H-1_A); 3.35 (1H, dd, $J_1 = 9.6$, $J_2 = 5.3$ Hz, H-1_B); 2.81 (1H, m, H-2); 2.53 [4H, m, (N–CH_2CH_3)$_2$]; 1.26 [26H, m, $(CH_2)_{13}$); 1.02 [6H, t, $J = 7.1$, (N–CH_2CH_3)$_2$]; 0.88, (3H, t, $J_1 = 6.8$, CH_3).). ^{13}C NMR (50.3 MHz, $CDCl_3$): δ (ppm) 138.7, 128.4, 127.8, 73.2, 70.7, 59.2, 44.3, 32.0, 29.8, 29.4, 29.0, 27.1, 22.8, 14.2. HRMS: Calcd: 404.3906 (M^++H); found: 404.3902. Anal. Calcd for $C_{27}H_{49}NO$: C, 80.33; H, 12.23; N, 3.47. Found: C, 80.31; H, 12.19; N, 3.45.
Analytical data for compound **8b**: Oil. IR ν_{max}: 3373, 2922, 2852, 1691, 1519, 1389, 1251, 1173 and 1053 cm^{-1}. ^1H NMR (200 MHz, $CDCl_3$): δ (ppm) 4.59 [1H, m, *NH*COOC$(CH_3)_3$]; 3.52 (1H, m, H-2); 2.50 [4H, q, $J = 7.1$, (N–CH_2CH_3)$_2$]; 2.34 (2H, d, J = 6.8, H-1); 1.24 [26H, m, $(CH_2)_{13}$); 1.44 [9H, s, NHCOOC$(CH_3)_3$]; 0.98 [6H, t, $J = 7.1$, (N–CH_2CH_3)$_2$]; 0.87, (3H, *t*, $J_1 = 6.5$, CH_3). ^{13}C NMR (50.3 MHz, $CDCl_3$): δ (ppm) 156.1, 78.9, 57.2, 49.4, 47.4, 33.8, 31.9, 29.7, 29.4, 28.5 25.8, 22.7, 14.2, 11.8. HRMS: Calcd: 413.4086 (M^+ + H); found: 413.4079. Anal. Calcd for $C_{25}H_{52}N_2O_2$: C, 72.76; H, 12.70; N, 6.79. Found: C, 72.75; H, 12.68; N, 6.73.
23. Rattan, A.; Kalia, A.; Ahmad, N. *Emerg. Infect. Dis.* **1998**, *4*, 195.
24. Whelen, A. C.; Felmlee, T. A.; Hunt, J. M.; Williams, D. L.; Roberts, G. D.; Stockman, L.; Persing, D. H. *J Clin. Microbiol.* **1995**, *33*, 556.
25. Siddiqi, S. *BACTEC-460 TB System, product and procedure manual. Revision E.* 1996. Becton Dickinson Mycrobioly Systems, Sparks, MD.